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Sensitivity of Dormant and Germinating *B. anthracis* Spores to Polycationic Compound

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This technical report has been reviewed and is approved for publication.

FOR THE DIRECTOR

//signed//
STEPHEN R. CHANNEL, DR-IV
Director, AF CBD Tech Base Programs
Air Force Research Laboratory

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14. ABSTRACT

The antimicrobial peptide protamine was found to be highly effective for rapid inactivation of *Bacillus anthracis* spores in aqueous suspension. Treatment of spores at the onset of germination with 10-, 100- and 1000-μg/ml protamine for 15 min at 37°C reduced viability by 80.9, 99.4 and 99.7%, respectively. Partially-germinated spores exposed to 10- or 100-μg/ml protamine became permeable to propidium iodide. Activity of protamine against dormant spores was a function of exposure temperature. While treatment of dormant spores with 1000-μg/ml protamine at ambient temperature did not significantly affect viability, dormant spores exposed to protamine at elevated temperatures were rendered susceptible to the toxicity of the peptide. Viability of dormant spores as a suspension subjected to 1000-μg/ml protamine at 55°C for 15 min was reduced by 99.5%. Protamine could be utilized as an effective enhancement of non-caustic treatments employed to process materials contaminated with the spore form of *B. anthracis*.

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1.0. INTRODUCTION

1.1. The spore phase of microorganisms provides a powerful natural defense that is integral to the survival of a variety of microbial species. As a dormant entity, the spore can survive for prolong periods of time in austere natural environments as well as tolerate the action of disinfectants or antimicrobial compounds. In comparison, vegetative cell forms of spore-forming microorganisms are usually less resistant to environmental stresses or antagonistic substances. The recalcitrance of spores enables the to persist in a wide variety of environments, creating practical problems such as food spoilage, degradation of industrial materials, and "sick building syndrome" resulting from colonization of ventilation systems.

1.2. While most spore-forming bacterial species are classified as non-pathogenic or opportunistically pathogenic, *Bacillus anthracis* is well-known a pathogen. As the causative agent of the lethal disease anthrax, health concerns of *B. anthracis* have traditionally been limited to the tanning industry and persons exposed to cattle materials that may harbor spores of the pathogen. More recently, *B. anthracis* has emerged as a biological agent of significance to national security and military defense due its potential utilization for weapons of mass destruction (Kahn, Sobel). The combination of the organism's pathogenicity and ability to survive in austere environments warrants concern about potential dissemination of the pathogen on the battlefield as well as a tool for acts of terrorism. Consequently, effective means of detection and disinfection are essential to control the pathogen for any threat scenario that could arise.

1.3. In this study, the naturally-occurring antimicrobial peptide protamine was examined for its effectiveness as an antimicrobial agent against *B. anthracis* spores. Protamine is a highly basic polycationic peptide with a broad spectrum of antimicrobial activity. Protamine is effective in disrupting the cell envelopes of both gram-positive and gram-negative bacteria. The permeabilizing action of protamine has been reported to occur rapidly, with inhibition of respiration and the efflux of cellular components occurring within minutes of exposure (Islam B, Johansen A, Johansen B). Treatment of cells with protamine has been reported to cause extensive cellular damage including formation of holes in bacterial cell envelops, blistering of cell membranes, condensation of the cytoplasm, and fusion of cells (Islam B, Johansen A). The disruptive effects of polycationic peptides such as protamine are believed to be due to their interaction with anionic cell wall components and anionic phospholipids (Gould, Johansen B). In addition to its bactericidal activity, protamine has been shown to be bacteriolytic for some microorganisms. Protamine and the cationic peptide polyarginine have been reported to cause the rapid lysis of *B. subtilis* vegetative cells (Antohi).

1.4. While the antimicrobial effect of protamine on gram-negative and gram-positive vegetative cells has been examined, there are few reports on the effects of this toxic peptide on spores. Protamine was reported to be inhibitory to the growth of *Bacillus subtilis* and *B. licheniformis* spores at concentrations ranging from 10- to 50-μg/ml protamine (Gould, Islam A). In this study, the antimicrobial activity of protamine against dormant and partially germinated spores of *B. anthracis* was examined.

2.0. MATERIALS AND METHODS

2.1. Spore production, purification, and germination. Spores of *Bacillus anthracis* ATCC strain 4229 were employed for investigation. While lacking pX01, which encodes the toxin complex, the strain harbors pX02 that encodes the capsule and, thus, possesses the wall components characteristic of the wild-type strain. Spores were produced by cultivation in Schaeffer's Sporulation Medium (Doi) for 24 to 48 h at 37°C with mixing (275 rpm). Spores were harvested by centrifugation at 6,000 X *g* for 10 min, washed twice with distilled water, and purified with four extractions using an aqueous two-phase separation system employing polyethylene glycol (Sacks). Purified spores were harvested by centrifugation at 10,000 X *g* for 10 min and washed twice with distilled water. Washed purified spore suspensions were stored in distilled water at 4°C for no more than one month prior to use for experiments.

2.1.1. Dormant spores were germinated in Nutrient Broth (Difco) supplemented with 150-mM L-alanine, hereafter referred to as germination medium. To produce partially-germinated spores for study, dormant *B. anthracis* spores were incubated in germination medium at 37°C with mixing (275 rpm) for 20 min prior to each experiment unless otherwise indicated. Partial germination was indicated by refractive index alteration as displayed by conversion of spores from bright to dark when observed by phase–contrast microscopy (1,000 X, oil). To examine the effect of protamine on spores at the inception of germination, dormant spores were mixed with protamine and germination medium simultaneously upon initiation of an experiment.

2.2. Protamine preparation. Protamine sulfate (Sigma Chemical Co.) was dissolved in distilled water as 10 mg/ml and filter sterilized using a cellulose acetate filter with 0.22-μm pore diameter. Solutions were prepared immediately prior to use.

2.3. Minimum Inhibitory Concentration (MIC) of protamine. The broth dilution format for MIC determination was employed to derive protamine concentrations that inhibit the growth of dormant spores as well as spores that were partially-germinated for 40 to 50 min prior to study. Duplicate two-fold dilutions of protamine in germination medium were inoculated with spores for final concentrations of protamine ranging up to 125 µl/ml and final spore densities of ca. 5×10^7/ml. Following incubation at 37°C with mixing (250 rpm) for 48 h, turbidity was evaluated by visible inspection to determine the lowest protamine concentration that completely inhibited growth. For each experiment, spores inoculated into germination medium that lacked protamine served as the postive control, while uninoculated germination medium served as the negative control. MIC values were based on averages of three trials.

2.4. Propidium iodide (PI) uptake by protamine-treated spores. Uptake of PI was employed to assess the effect of protamine on the integrity of the spore wall. While intact walls of healthy spores exclude PI from the organism, spores with disrupted walls are permeable to the compound and can be observed by fluorescence microscopy. Spores partially germinated for 20 min were employed to determine the effects of protamine on permeability to PI. Since the germination process may release spore constituents that could interfere with the activity of protamine, evaluations were conducted with partially-

germinated spores suspended in original germination medium as well as partially-germinated spores washed with and suspended in fresh germination medium. Spore suspensions dosed with 10-μg/ml protamine and 1-μg/ml PI (Molecular Probes, Inc., Eugene, OR) were incubated at 37°C in darkness for 1 h. Aliquots (8 μg) were delivered to a Petroff-Hausser counting chamber (1/400-mm^2 ruling pattern, 0.02-mm depth; Hausser Scientific, Horsham, PA) to count total spores by phase-contrast and PI-stained spores by epifluorescence (excitation 510 to 550 nm, emission 590 nm) microscopy at 400 X magnification. Total spore and fluorescent spore counts were employed to derive the percentage of PI-stained spores for protamine-treated and untreated control suspensions.

2.5. Inactivation of spores by protamine. The effects of protamine on viability of dormant spores as well as spores in the process of germination were determined on the basis of cultivability on growth medium. Suspensions adjusted to a density of ca. 10^7 spores/ml were dosed with protamine (0-, 10-, 100-, or 1,000-μg/ml), incubated at a defined temperature (ambient, 37°C, or 55 °C) with mixing (250 rpm), and sampled periodically during the protamine exposure. Samples were diluted in phosphate buffer (50-mM, pH 7.0) and plated in triplicate on Nutrient Agar and incubated at 37°C for 24 to 36 h for development of colonies. Inactivation was presented as the logarithm of colony-forming-unit density (cfu/ml) as a function of time, with the initial value being cfu/ml prior to protamine dosing. To evaluate dormant spores, precautions were taken to ensure the organisms maintained their dormancy during testing. This was accomplished by suspending dormant spores in distilled water for protamine exposure and diluting samples in phosphate buffer (50-mM, pH 7.0) for plating. To test spores at the inception of germination, dormant spores were suspended in germination medium and dosed with protamine simultaneously. To examine partially-germinated spores, dormant spores were suspended in germination medium for 20 min prior to dosing with protamine.

2.6. Effect of temperature on inactivation of dormant spores by protamine. The influence of temperature on the sensitivity of dormant spores to protamine was determined by employing the inactivation procedure described in the preceding section with exposures at various temperatures. Viability based on cultivability was assessed for treatment with 1,000-μg/ml protamine at ambient temperature, 37°C, and 55°C. In addition, the inactivation of spores at the highest test temperature (55°C) was compared following exposures to 10, 100-, and 1,000-μg/ml protamine.

2.7. Effect of $MgCl_2$ on the action of protamine against dormant spores. Because divalent cations such as Mg^{2+} and Ca^{2+} have been reported to reduce the effectiveness of protamine on gram-positive and gram-negative vegetative cells (Johansen C), the effect of $MgCl_2$ on the action of protamine against *B. anthracis* spores was examined. Dormant spores, which were suspended in distilled water containing 1000-μg/ml protamine, were incubated at 50°C for 20 min and then treated with $MgCl_2$ (10-, 20-, 50-, 100-, and 200-mM). Following the cation addition, incubation of suspensions at 50°C was continued for up to 3.75 h. To evaluate the effect of $MgCl_2$ on spore cultivability, aliquots of the suspensions were collected before protamine addition, immediately prior to MgCl2 addition, and at various times after cation addition. To enumerate densities of viable spores, aliquots were serially diluted in potassium phosphate buffer (50-mM, pH 7), plated in triplicate on Nutrient

Agar, and incubated at 37°C for 24 to 36 hours for development of colony forming units. The controls included: (1) spore suspensions dosed with protamine without $MgCl_2$, and (2) untreated spore suspensions that lacked either protamine or $MgCl_2$. The dormant state of the spores was maintained during the evaluation by suspension of the organisms in distilled water or buffer prior to cultivation on growth medium.

3.0. RESULTS

3.1. Minimum inhibitory concentrations. Low concentrations of protamine inhibited growth of both dormant and partially-germinated *B. anthracis* spores that were incubated in germination medium amended with the antimicrobial compound. The minimum inhibitory concentration (MIC) of protamine was 12.5-μg/ml for spores that were dormant prior to inoculation and 15.6-μg/ml for spores that were partially germinated prior to inoculation. These MIC values for *B. anthracis* spores are comparable to the protamine MIC values reported for spores of other *Bacillus* species (Gould).

3.2. Uptake of propidium iodide. The disruptive action of protamine on the spore wall was evidenced by the enhanced uptake of propidium iodide (PI) by partially-germinated spores treated with the peptide. Addition of protamine (10-μg/ml) to a suspension of partially-germinated spores in germination medium increased the proportion of PI-stained organisms by 54.3% following incubation with the antimicrobial at 37°C for 1 h (Table 1). Partially-germinated spores, which were washed with and suspended in fresh germination medium prior to protamine exposure, incorporated PI more efficiently. The washed-spore proportion (82.3%) rendered permeable to PI due to protamine treatment exceeded the unwashed-spore proportion (54.3%) that stained with PI as a result of protamine exposure (Table 1).

	Percent PI-permeable spores[1]		Percent PI-permeable spores due to protamine[2]
	Protamine treated	Untreated control	
Unwashed spores[3]	60.3 (± 14.3)	13.1 (± 11.9)	54.3
Washed spores[3]	85.0 (± 6.8)	15.4 (± 9.8)	82.3

[1] Percent PI-permeable spores relative to total spores was determined by direct counting using epifluoresence and phase-contrast microscopy with a Petroff-Hauser counting chamber.
[2] [(% protamine-treated PI-permeable spores) − (% control PI-permeable spores)] ÷ [100 − % control PI-permeable spores] x 100 %.
[3] Spores subjected to germination conditions for 20 min were in original germination medium or washed with and suspended in fresh germination medium immediately prior to protamine treatment.

Table 1. Effect of protamine (10-μg/ml) treatment at 37°C for 1 h on propidium iodide (PI) permeability of washed and unwashed partially-germinated spores

3.2.1. Sensitivity of partially-germinated spores to protamine was a function of the concentration of antimicrobial compound during exposure. Treatment with 100-μg/ml protamine at 37°C for 30 min increased the proportion of organisms permeable to PI by 94.7%. This increase in PI permeability exceeded that observed with the lower protamine concentration (10-μg/ml) and longer exposure (1 h) as displayed in Table 1. In contrast to the lower-level protamine exposure, the effect of 100-μg/ml protamine treatment on PI uptake was not significantly different when comparing unwashed spores with washed spores.

3.3. Spore viability following protamine treatment. Of the various spore states examined, spores partially germinated for 20 min were the most sensitive to the antimicrobial. Following 15 min of exposure to 10-, 100-, and 1,000-μg/ml protamine, levels of cultivable organisms were reduced by 80.9, 99.4, and 99.7%, respectively (Figure 1A). Following rapid inactivation during the first 20 min of exposure at the two higher protamine concentrations, densities of cultivable spores approached a plateau representing an approximate 3-logarithm reduction in viable organisms. In comparison, spore inactivation by 10-μg/ml protamine was less pronounced as indicated by an approximate 1-logarithm decrease in viable organisms following 120 min of exposure.

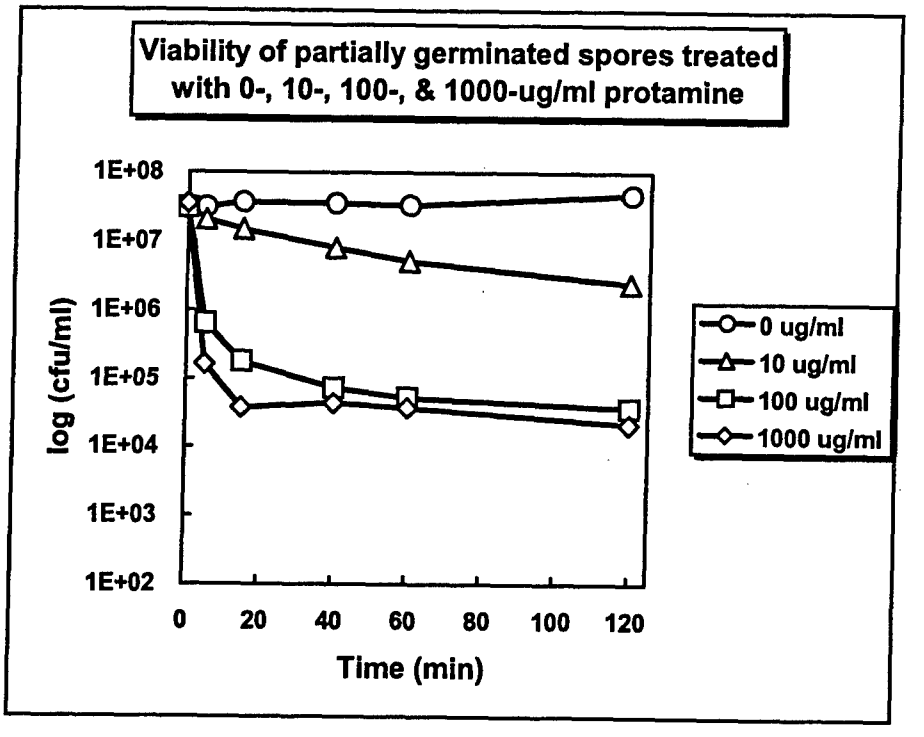

Figure 1A. Survival of *B. anthracis* spores that were germinated for 20 min prior to exposure in the presence of 0-, 10-, 100-, and 1,000-μg /ml protamine.

3.3.1. Spores exposed to protamine at the inception of germination were effectively inactivated, although not as extensively as spores that had been in the process of germination for 20 min prior to being dosed with the peptide. Following 15 min of exposure to 100- and 1,000-μg/ml protamine, levels of cultivable spores decreased by 86% and 97%, respectively (Figure 1B). Logarithmic decrease in cultivable spores following 60 min of exposure reached approximately 1.5 and 2 for 100- and 1,000-μg/ml protamine, respectively. As observed with partially-germinated spores, spores at the inception of germination exposed to 10-μg/ml protamine were considerably less affected by the antimicrobial than spores exposed to the compound at either of the higher concentrations.

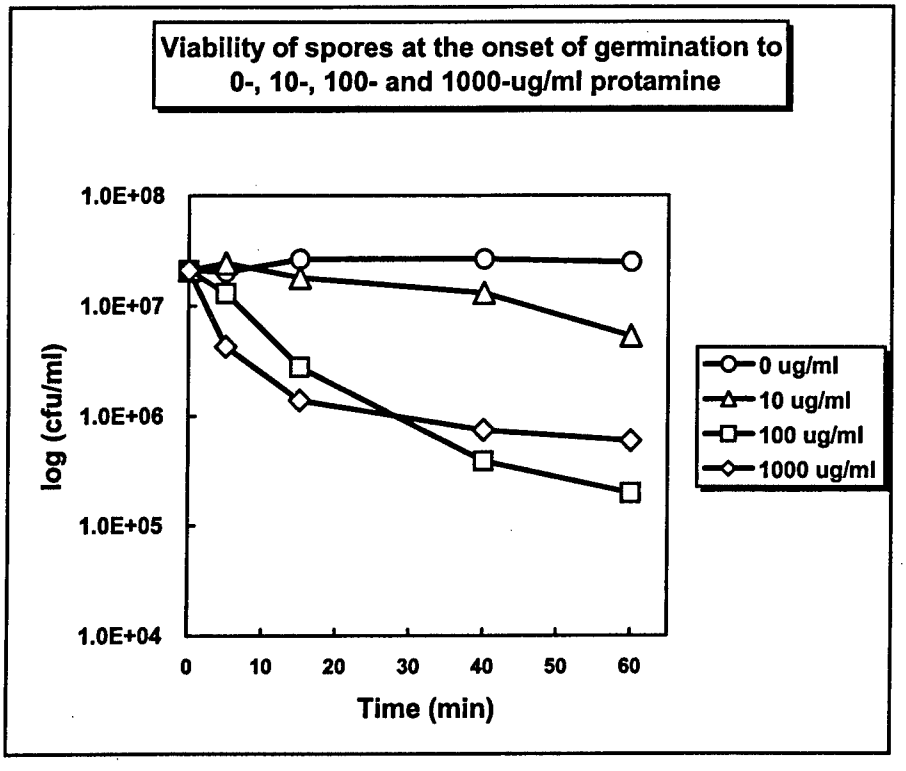

Figure 1B. Survival of *B. anthracis* spores simultaneously exposed to protamine and a germinant in the presence of 0-, 10-, 100-, and 1,000-μg /ml protamine.

3.3.2. Dormant spores, which were exposed to protamine at ambient temperature, maintained levels of viability comparable to that of the control dormant spores not subjected to the antimicrobial (Figure 1C). The extent of the tolerance was demonstrated by cultivability of spores following exposure to the highest protamine level tested (1,000-μg/ml). However, elevated temperatures greatly potentiated the efficacy of protamine treatment. The cultivability of dormant spores exposed to 1,000-μg/ml protamine for 1.8 h at 37°C and 55°C decreased by approximately 1.5 and 5 logarithms, respectively (Figure 2). A dose effect was demonstrated with dormant spores exposed to protamine at 55°C (Figure 3). Reduction in cultivable spore density was much greater with 1000-μg/ml protamine exposure than with 100-μg/ml antimicrobial. Exposure to the lowest protamine level (10-μg/ml) at the

elevated temperature did not alter dormant spore viability. Control dormant spores, which were suspended in distilled water without protamine, maintained stable levels of viability at the highest test temperature (55°C) for the 4-h duration of the experiment (Figure 2).

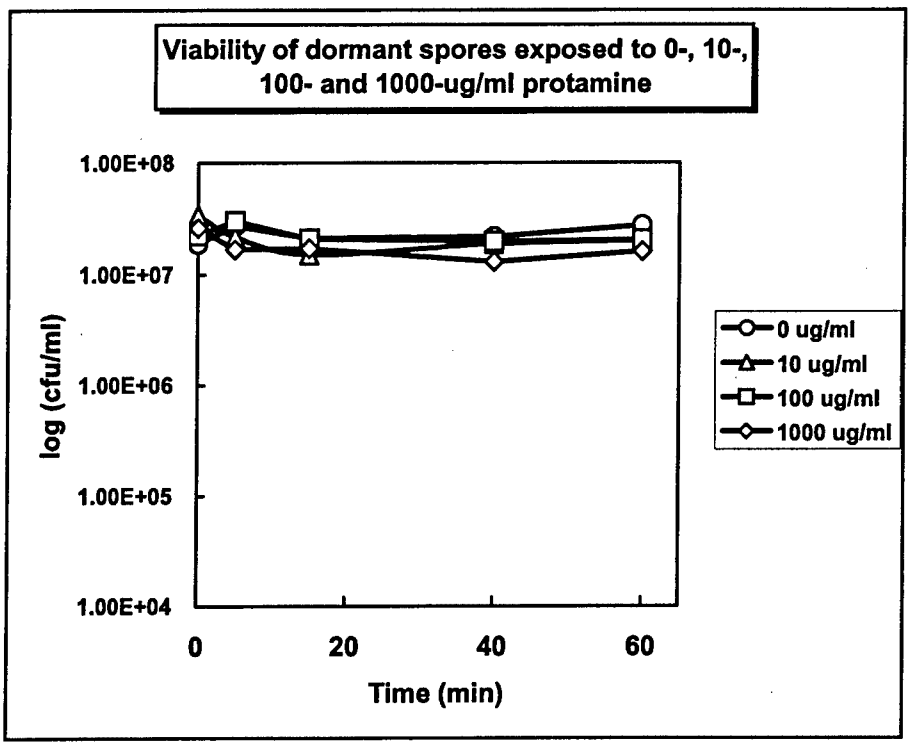

Figure 1C. Survival of dormant *B. anthracis* spores in the presence of 0-, 10-, 100-, and 1,000-μg /ml protamine.

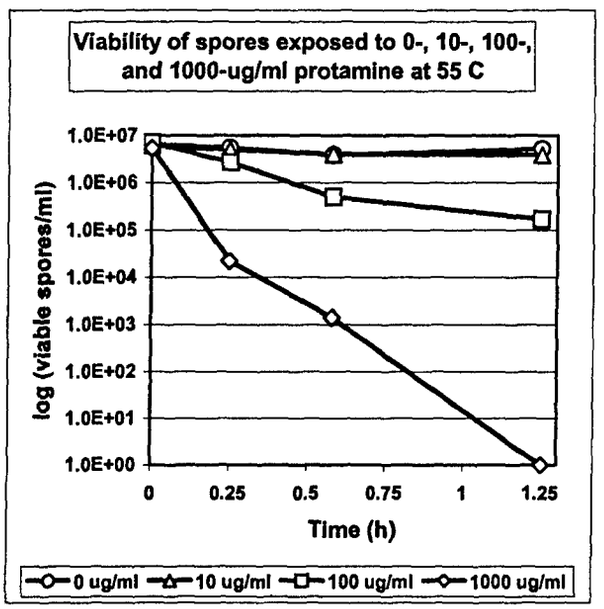

Figure 2. Viability of spores exposed to 0-, 10-, 100-, and 1000-μg /ml protamine at 55°C.

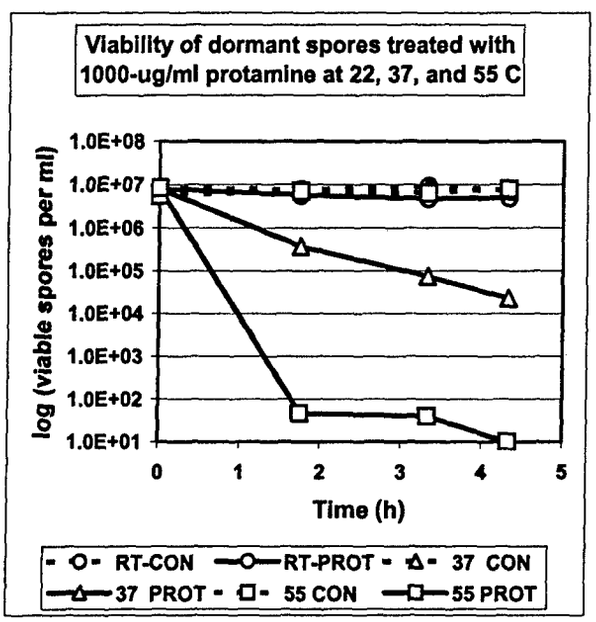

Figure 3. Viability of spores treated with 1000-μg /ml protamine at 22, 37, and 55°C.

3.4. Effect of divalent cations on protamine treatments. Since binding of protamine to gram-positive and gram-negative bacterial cells was reported to be reversible with divalent cations such as Mg^{2+} and Ca^{2+} (Johansen C), the influence of Mg^{2+} on the antimicrobial activity of protamine on the bacterial spore was investigated. While the cultivability of dormant *B. anthracis* spores suspended in distilled water was reduced by exposure to1000-μg/ml protamine at 55°C, the effect of the antimicrobial was negated by addition of $MgCl_2$ (Figures 4 and 5). Without amendment with the divalent cation, the proportion of spores that remained cultivable decreased by at least two logarithms following 20 min protamine exposure. Following extended protamine exposure (1.5 to 3.8 h), the level of cultivable spores decreased by more than 5 logarithms. However, the presence of $MgCl_2$ terminated the effect of protamine on spore viability. Upon addition of the cation following 20 min protamine exposure, reduction in the number of cultivable spores ceased and the level of viable spores remained constant for the duration of the antimicrobial exposure. A dose effect was not observed at the $MgCl_2$ concentrations tested. The magnitude of the effect of the divalent cation on negating the action of protamine on spore cultivation was similar at lower (10- and 20-mM) or higher (50-, 100-, and 200-mM) $MgCl_2$ concentrations.

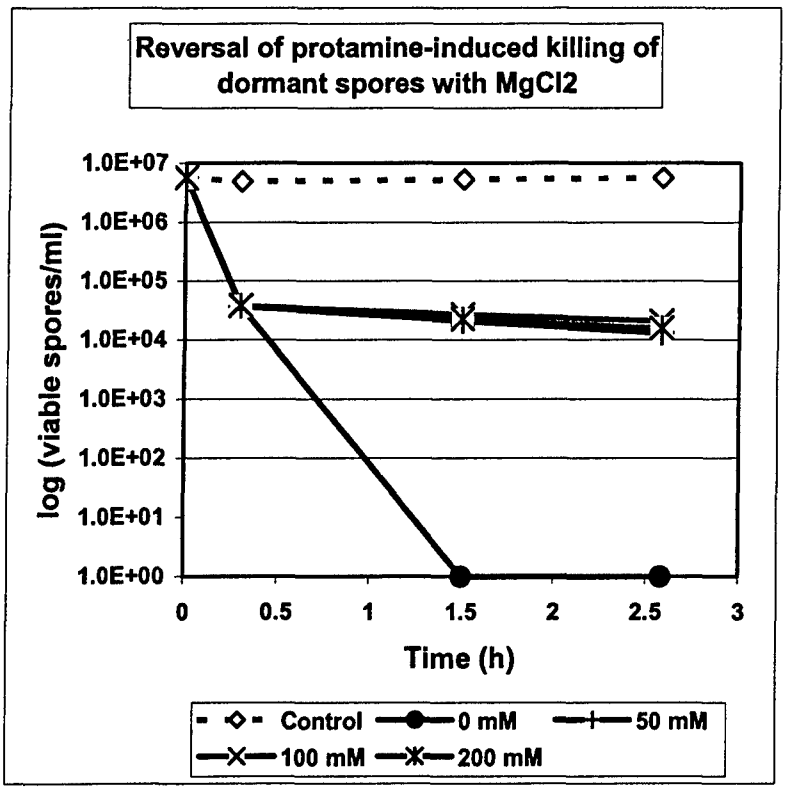

Figure 4. Influence of 0-, 50-, 100- and 200-mM $MgCl_2$ on the antimicrobial activity of protamine against dormant spores.

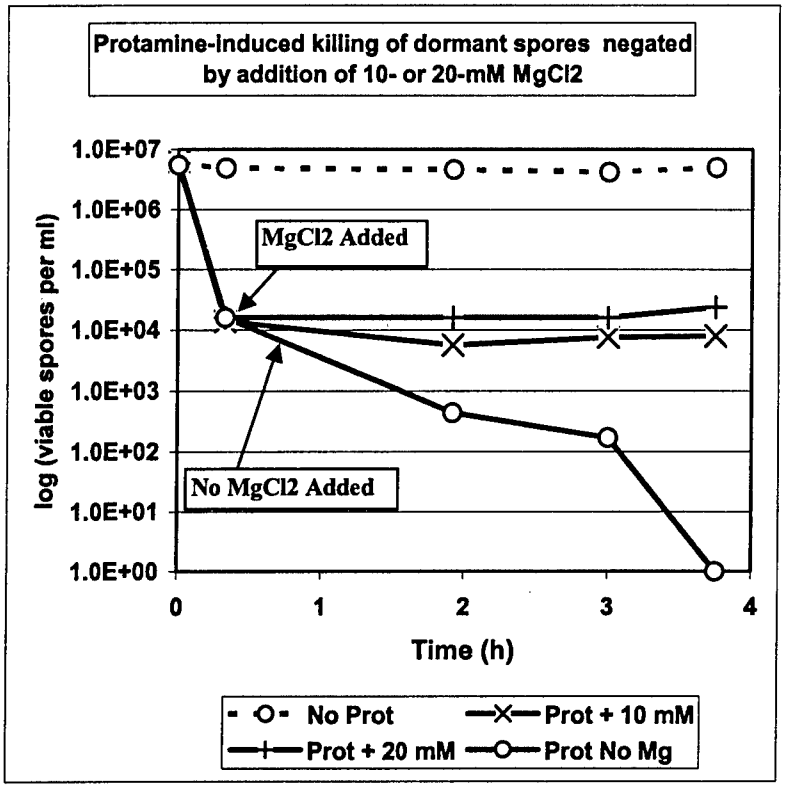

Figure 5. Influence of 0-, 10- and 20-mM $MgCl_2$ on the antimicrobial activity of protamine against dormant spores.

4.0. DISCUSSION

4.1. This is the first reported investigation of the effect of protamine on the spore of *Bacillus anthracis*. Basic results of our study indicate that sensitivity to the antimicrobial compound was greater for spores in the process of germination. In contrast, spores in the dormant state were more tolerant to the action of protamine. This finding is consistent with the general ability of the bacterial spore to survive adversity. A microorganism existing as a dormant spore can be remarkably tolerant to a variety of harsh physical and chemical conditions and, thereby, persist in inhospitable environments.

4.2. In this study, protamine-induced alteration of spore wall permeability was not demonstrated with spores in the dormant state at ambient temperature. Dormant spores exposed to the peptide remained impermeable to propidium iodide. However, once germination was engaged, spores treated with a low level of protamine incorporated the DNA-intercalating compound. Alteration of the organism during transition to the vegetative cell could yield wall structures that are accessible to or sensitive to the effects of the

polycationic compound. The effect of protamine on the permeability of the cell wall of gram-positive, as well as gram-negative, bacterial species has been reported (Johansen C).

4.3. Sensitivity of the germinating spore to protamine treatment, as demonstrated by permeability to propidium iodide, was promoted by suspending the organism in fresh germination medium. The proportion of partially-germinated spores incorporating propidium iodide following protamine treatment was greatly increased for spores washed with and suspended in unused broth prior to protamine exposure. This observation could be attributed to wall components or other materials released during germination that quench protamine. During the germination process, a significant portion of the spore coat constituents of the spore is released from the organism. Comprising about 30% of the spore dry weight, released components predominately include calcium, dipicolinic acid, fragments of depolymerized murein as well as small amounts of amino acids, small peptides, and proteins (3). Reaction of protamine with released biological materials could reduce the concentration of available antimicrobial and, thus, diminish the effect of the protamine dose on the organism. In addition, released biological materials could obstruct the availability of protamine at the surface of the organism and protect target sites of the wall from the action of the antimicrobial.

4.4. In addition to altering permeability, protamine exposure affected *B. anthracis* viability as ascertained by cultivability on growth medium or in broth. However, as observed during the evaluation of protamine's action on permeability, effects of the antimicrobial on viability were strongly influenced by the physiological state of the spore. Dormant spores suspended in water to maintain dormancy tolerated extreme protamine exposures. Dormant spores, which were subjected to 1,000-μg/ml protamine for one hour at ambient temperature, were fully cultivable on growth medium as compared with control spores not exposed to the peptide. While dormancy appeared to impart tolerance to protamine, spores in the process of germination were sensitive to the action of the compound. Spores exposed to protamine while suspended in germination medium were inactivated as displayed by loss of cultivability on growth medium. In addition, the extent of germination appeared to influence the degree of sensitivity to the antimicrobial. Inactivation was more pronounced for spores engaged in germination for 20 minutes prior to protamine exposure. In contrast, spores dosed with protamine at the initiation of germination were less sensitive to the compound. Differences in protamine sensitivity as a function of germination time were displayed by inactivation rates. During the initial 5 minutes of exposure to protamine (100-μg/ml), the inactivation rate was about 6-fold greater for spores exposed to protamine following 20 minutes of germination than for spores exposed to protamine at the onset of germination. These findings suggest that protamine acts primarily on the spore actively engaged in germination and not when the dormant spore is activated to transition from the dormancy to germination. Once fully engaged in the germination process, the spore wall is significantly altered and could be more susceptible to the action of protamine due to the appearance of sites susceptible to the action of the compound. In addition, degeneration of the wall permeability barrier that occurs during germination (Gould) may allow entrance of protamine into the cell and attack of internal sites such as nucleic acids and other negatively charged biomolecules.

4.5. Following activation of the dormant spore and the subsequent germination process, the final event that completes the transition of the spore to a vegetative cell is outgrowth of the spore. Protamine's action on the entire sequential process was evaluated by determining the lowest protamine concentration that prevented dormant spores suspended in germination broth from germinating, outgrowing, and finally multiplying as vegetative cells to a high density. The minimal inhibitory concentration (MIC) of protamine (12.5-μg/ml) that prohibited dormant spores suspended in broth from becoming an actively growing cell culture was within the range of minimal concentrations (10- and 50-μg/ml) reported to inhibit growth of *B. subtilis* and *B. licheniformis* spores suspended in broth (Gould). For spores in the process of germination when dosed with protamine, a higher MIC (16.5-μg/ml) was required to prevent the spores from progressing to actively growing culture of cells. This finding contrasts findings of our other assays that indicated germination enhanced the sensitivity of the spore to the action of protamine. The discrepancy could be attributed to the release of materials from the spore during germination that quench protamine and, thus, reduce the antimicrobial's effective concentration. Spores dosed with protamine at dormancy would be subjected to the full concentration of the antimicrobial up to the point of germination when spore materials are released. In contrast, a broth suspension of spores fully engaged in germination would contain released materials that would quench and reduce the effective protamine at the instant of protamine addition. Islam *et al.* (A) reported spore sensitivity to protamine was affected by growth medium constituents and the spore concentration. At higher spore densities, protamine treatments were reported to be less effective presumably due to the non-specific binding of protamine with either organic matter or anionic substances in the medium.

4.6. The tolerance of the *B. anthracis* spore to protamine was only demonstrated when the pathogen was in its dormant state during exposure to the antimicrobial. However, this tolerance was only observed for exposures conducted at ambient temperature. In contrast, dormant spores treated with the compound at elevated temperatures succumbed to the effect of the antimicrobial. While dormant spores suspended in water remained fully cultivable during exposure to 1,000-μg/ml protamine at ambient temperature, the proportion of cultivable dormant spores exposed to the same level of antimicrobial at 55°C for only 15 minutes was reduced by 2.5 logarithms. With an inactivation rate approximating linearity for more than an hour, the antimicrobial exposure at 55°C for 1.25 hour reduced the density of cultivable spores by almost 7 logarithms. Combined heat and protamine treatment to increase the effectiveness of the antimicrobial for inhibiting spore growth has been reported (Islam B). Protamine sensitivity of the dormant spore at 55°C could be attributed to activation of the spore by heat. Although spores are activated more efficiently in the presence of nutrients such as glucose or peptone, dormant spores suspended in water can be activated by heat (Gould). Once activated into the germination phase, the spores would be sensitized to protamine as demonstrated in this study. Protamine treatment augmented with heat could provide the basis for effective means to inactivate the dormant *B. anthracis* spore. While 55°C significantly enhanced the efficacy of protamine, higher temperatures or addition of other compounds known to activate the dormant spore could be employed to devise highly effective treatments to kill the dormant spore.

4.7. The mode of action of protamine, as a polycationic compound, against the *B. anthracis* spore could involve charge interations between the antimicrobial and the spore structure. The disruptive effect of protamine and poly-L-arginine on the wall of the gram-negative bacterial cell is attributed to the action of the polycationic compounds on the charge interactions between divalent cations and negatively-charged lipopolysaccharide molecules that stabilize the outer membrane structure.

4.8. Reversal of protamine binding to gram-positive and gram-negative vegetative cells by $MgCl_2$ has been reported (Johansen C). In this study, the addition of $MgCl_2$ terminated the inactivation of dormant *B. anthracis* spores by protamine at 55°C. Upon addition of the divalent cation 20 minutes after the initiation of the inactivation, inactivation of the dormant spore as assessed by cultivability on growth medium was immediately terminated. In addition, negation of the antimicrobial activity remained constant for the remainder of the exposure. The profound effect of $MgCl_2$ suggests the antimicrobial action of protamine involves charge interactions, possibly at the surface of the *B. anthracis* spore. Protamine, as a polycationic molecule, may displace divalent cations such as Ca2+ or Mg2+ that stabilize the spore wall and, consequently, disrupt the wall structure. Neutralization of the effect of protamine by addition of $MgCl_2$ could be attributed to cations displacing polycationic molecules associated with the spore wall or quenching unbound protamine.

4.9. Use of protamine as a biological alternate to chemical preservatives or disinfectants has been proposed on the basis of the compound being naturally-occurring, nontoxic to humans, and effective against a broad spectrum of microorganisms (Johansen, 1997; Hansen). Due to stability at elevated temperatures and minimal human toxicity, utility of protamine as well as other polycationic compounds as food additives to control contamination by microbial spores has been proposed. Spores of various species of *Clostridium* and *Bacillus* are of concern to the food industry due to their potential for spoilage of or toxin production in improperly processed foods. In addition, potential for deliberate contamination of food supplies or facilities with biological agents is an issue relevant to homeland security and military defense (Sobel). Possible release of biological agents via overt or covert actions as well as collateral contamination warrants the need for novel means to ensure food safety during unconventional or unpredictable situations. Due to its ability to maintain viability during adverse conditions and to cause human disease through ingestion, the *B. anthracis* spore is a potential food-borne threat agent. Based on findings of this study demonstrating the activity of protamine against the *B. anthracis* spore, the antimicrobial may have potential utility as a food additive that kills the pathogen. Although protamine did not inactivate the dormant spore at ambient temperature, the pathogen was killed when exposed to the compound at 37 or 55°C. Thus, spore-contaminated food amended with protamine and heated at mild temperatures for sufficient time could be rendered safe for consumption. However, the practical application of protamine as a food additive to inactivate the food pathogen would require further investigation of parameters such as effectiveness at temperatures exceeding 55°C and as an additive to various types of foods.

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